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Highly sensitive determination of copper in HeLa cell using capillary electrophoresis combined with a simple cell extraction treatment

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ABSTRACT

A new separation system of capillary electrophoresis (CE_1) for the highly sensitive determination of copper was established by using ethylenediaminetetraacetic acid (EDTA) as a complexing agent and employing cetyltrimethylammonium chloride (CTAC) as a capillary inner wall modifier. Benefitted from the combination of field-enhanced sample injection (FESI) method, a limit of detection (LOD) of 2.7 nM was obtained, which was much lower than that of the conventional methods. This made it possible to determine trace copper in HeLa cell only by a simple cell extraction (CE_2) treatment. Two copper-extraction methods—acid-hydrolysis and freeze–thaw—were compared. Limited by the requirement of low ion strength in FESI, only the extract using freeze–thaw could be successfully applied in the determination. The effectiveness assessment of this CE^2–FESI method was adopted by inductively coupled plasma-atomic emission spectrometry (ICP-AES) as a gold standard.

1. Introduction

The development of life science has increasingly found the importance of copper to people's health. It is reported that on average a cancer cell has 46% more copper than that a normal cell [1]. The Wilson disease and the Menkes disease are found as disorders of copper transportation [2,3]. Hence, due to the various roles of copper in biological systems, it is important to find a method to monitor copper level in cells. Some methods are already developed for the detection of metal ion, such as ICP-AES [4,5], atomic absorption spectrometry (AAS) [6,7], chemiluminescent detection [8,9] and mass spectrometry (MS) [10], and some methods have already been used to detect copper in living cells [11–13]. Nevertheless, many of them need complex sample pretreatment steps or pre-separation steps, which are often tedious and time consuming.

Recent research has proven the capillary electrophoresis (CE_1) method to be a powerful tool to separate and determine metal ions. A series of detection methods, such as ICP [14,15], chemiluminescent [16], ultraviolet (UV) [17,18], electrochemical detection (ECD) [19] and fluorescence [20] are used to detect the ions after the separation process. Among them, the direct UV detection of metal ions with the help of complexing agents is mostly used. Some common complexing agents, such as α-hydroxyisobutyric acid [21], EDTA [22], cyclohexanediaminetetraacetic acid (CDTA) [23] and *o*-phenantroline [23], are used in the direct UV detection. To improve the sensitivity of detection, the sample stacking technique such as field-enhanced sample injection (FESI) can be used to make the metal ions online concentrated [24].

Unfortunately, there are few references about the detection of copper in cell [25,26], and there is no report regarding detecting copper in cell using CE_1 until now, though the high-resolving power of the CE_1 method and the possibility for low detection limits make the approach particularly appealing. Actually, instead of free ion, the copper in cell is mostly combined with protein, which makes the extraction and determination of copper in cell challenging. In this study, the trace copper in cell is determined by using a simple cell extraction and capillary electrophoresis combined with field-enhanced sample injection (CE^2–FESI).

2. Materials and methods

2.1. CE_1 apparatus

All CE_1 experiments were performed with a P/ACE MDQ instrument equipped with a diode-array UV detector (Beckman Coulter, USA). UV detection was carried out at 260 nm. Data acquisition and instrument control were carried out using 32 Karat software (Version 8.0). Separations were performed in 50.2 cm (40.0 cm to detector) uncoated fused-silica capillaries with 75 μm i.d. and 363 μm o.d. (Polymicro Technologies, USA).

2.2. CE_1 procedure

Before the first run, the new capillary was flushed with methanol for 5 min, pure water for 2 min, 1.0 M HCl solution for

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5 min, pure water for 2 min, 1.0 M NaOH solution for 10 min, pure water for 2 min in sequence. The capillary was finally equilibrated with running buffer for 15 min. To improve the reproducibility, the capillary was flushed with 1.0 M NaOH solution, pure water and running buffer for 3 min between consecutive analyses. In FESI, the injection was performed electrokinetically after a water plug at 0.5 psi for 10 s. All measurements were carried out at least three times. The coolant temperature was controlled at 25 °C.

2.3. Reagents

LL3-31, which was also known as di-2-pyridyl thiosemicarbazone, was obtained from Lin Group. Copper sulfate and EDTA were purchased from Beijing Chemical Works (Beijing, China). CTAC was purchased from J&K Chemical (Beijing, China). Fetal bovine serum was purchased from Gibco (New York, USA). DMEM (Dulbecco's Modification of Eagle's Medium) and trypsinase were purchased from Solarbio (Beijing, China). Buffers and solutions were prepared with pure water, which was purchased from Wahaha Group Co. Ltd. (Hangzhou, China). All other chemicals were of analytical reagent grade and used without further purification. Cu-LL3-31 (1.00 mM) that acts as a Cu-spiking drug in cells in this system was prepared by mixing 10 μL 100 mM LL3-31, 50 μL 20.0 mM $CuSO_4$ and 940 μL H_2O.

2.4. Cells culture, Cu-spiking and extract

HeLa cells were cultured in DMEM with 10% fetal bovine serum added. The cells were maintained in 10 cm dishes at 37 °C in the incubator until harvesting for the experiment. When harvesting, the DMEM was drawn out from the culture dishes, and the dishes were rinsed twice with 5 mM NaCl solution and then treated with 1 mL trypsinase (EDTA added) for 2 min in the incubator. After that, 2 mL NaCl solution was added into the centrifuge tubes in order to wash the cells. The cells were then centrifuged at 3000 rpm for 5 min, while the supernatants were discarded. Finally, the cells were kept frozen at −80 °C until extracting.

To assess the accuracy of the proposed CE^2–FESI method, Cu-spiking to the blank cells is necessary. When the cells were nearly fully grown in the dishes, 20 μM Cu-LL3-31 solution was added into the dishes. Then the cells were maintained at 37 °C in the incubator for about 6 h. With the help of a light microscope, it was easy to observe that the morphology of cells could be influenced by the Cu-spiking drug, from spindle shape to circular shape. At this moment, the cells were harvested as the blank cells harvesting procedure.

Cell extract is extremely important in this system. The two methods, acid-hydrolysis and freeze–thaw, were used to extract copper out of the cells. Their extraction procedures are described in Fig. 1 in detail.

3. Results and discussion

3.1. Effect of applied voltage

In general, EDTA formed a negative charged chelate $[Cu–EDTA]^{2-}$ with Cu^{2+}. Meanwhile, the electroosmotic flow (EOF) is in the

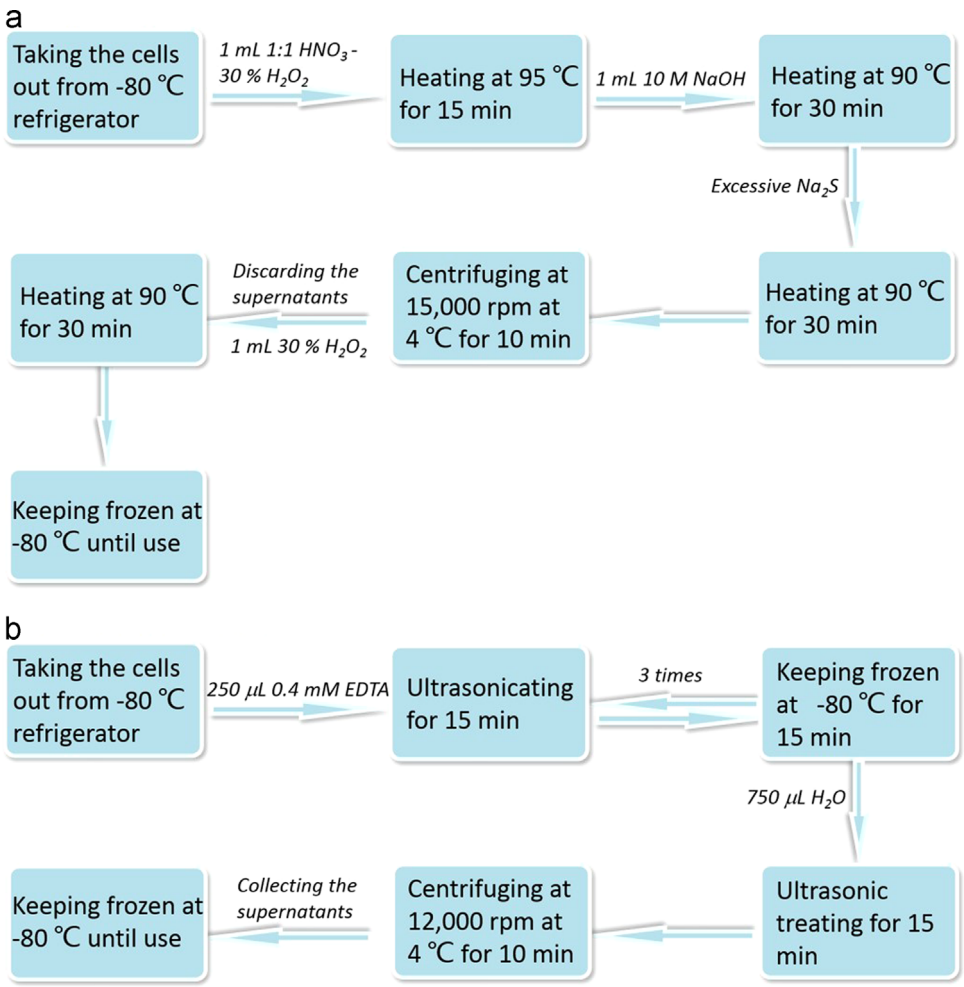

Fig. 1. The extraction procedures of acid-hydrolysis method (a) and freeze–thaw method (b).

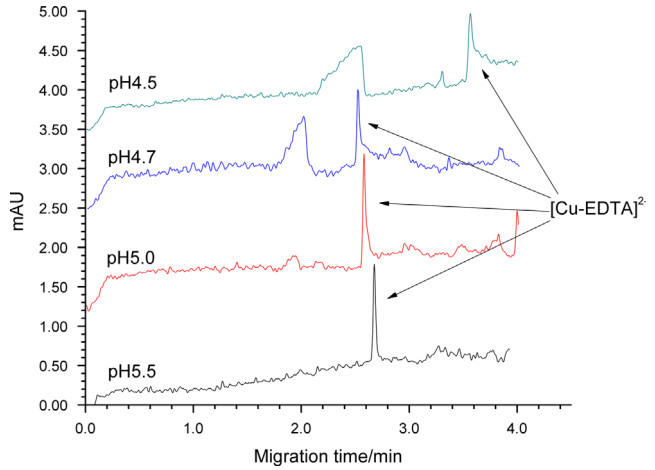

Fig. 2. Effect of the pH value of running buffer. Sample solution: 100 nM $CuSO_4$ mixing with 1 μM EDTA. Experimental conditions: running buffer, 50 mM ABS mixing with 0.3 mM CTAC; injection by voltage, −2 kV for 5 min; water plug, 0.5 psi for 10 s; separation voltage, −20 kV.

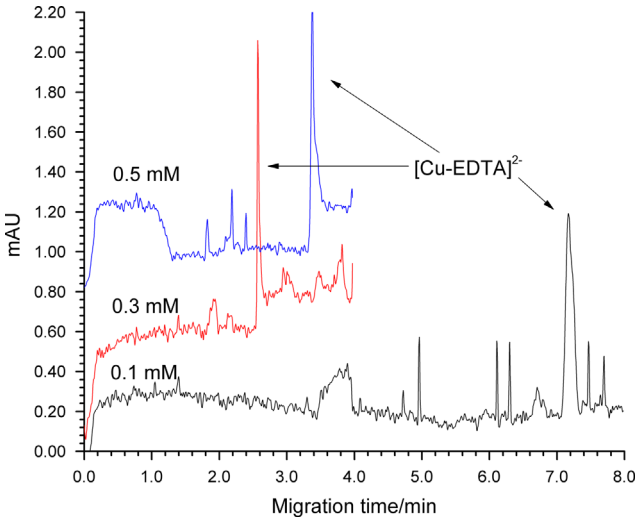

Fig. 3. Effect of the concentration of CTAC. Running buffer: 50 mM ABS (pH=5.0) mixing with various concentrations of CTAC. Sample solution and other experimental conditions the same as in Fig. 2.

opposite direction to the electrophoretic mobility of $[Cu–EDTA]^{2-}$, due to the silanol groups in the inner wall of the capillary. 50 mM acetate buffer solution (ABS) is chosen as the running buffer according to Ref. [18]. Under such a weak acid system, if a positive voltage was applied as usual, a lower EOF would bring about a longer migration time and a wide $[Cu–EDTA]^{2-}$ peak. So a negative voltage combined with a cationic surfactant as an EOF modifier was applied in this study.

3.2. Effect of pH

The pH has great impact on EDTA chelating with Cu^{2+} and hence the stability of chelate. The effect of the four pH values to the detection of $[Cu–EDTA]^{2-}$ is shown in Fig. 2. With the decrease of pH in the running buffer, the peak of $[Cu–EDTA]^{2-}$ tailed more and more obviously, and the peak heights decreased. This could be explained by the fact that when the pH value of the buffer decreased, the increase of the H^+ concentration made a big acid effect of EDTA, which disturbed the complexation of Cu^{2+} and EDTA, especially in the case of pH 4.5. From the distribution species-pH diagram of Cu–EDTA complex, the $[Cu–EDTA]^{2-}$ complex could be stable when the pH value of running buffer was above 5.0. Considering that when the pH value was above 5.5, there might not be enough buffering capacity for ABS, the pH value of the buffer was finally chosen as 5.5.

3.3. Effect of CTAC

When there was no cationic surfactant in the running buffer, the $[Cu–EDTA]^{2-}$ would run toward the detector as the electrophoretic mobility of $[Cu–EDTA]^{2-}$ was lower than the EOF. But due to the lower pH value of running buffer, the slower EOF would make a longer migration time to the detector for $[Cu–EDTA]^{2-}$. As a direct result, the longer migration time brought about a serious diffusion of $[Cu–EDTA]^{2-}$ and hence the wider and lower peak. However, after adding cationic surfactant such as CTAC into the running buffer, the EOF was reversed to the same direction as the electrophoretic mobility of $[Cu–EDTA]^{2-}$ when the inner wall of capillary was fully coated by the surfactant. This would make the migration time of $[Cu–EDTA]^{2-}$ extremely shorter, leading to a narrower and higher peak. Consequently, adding CTAC into the running buffer could improve the detection. In the experiment, 0.1 mM, 0.3 mM and 0.5 mM CTAC were added into the running buffer to observe their effect, respectively, and the results are shown in Fig. 3. When the concentration of CTAC was 0.1 mM, the migration time of $[Cu–EDTA]^{2-}$ was slower than that in the other two buffers, showing that the inner wall of the capillary might not be fully coated by the CTAC cation. When the concentration of CTAC was increased to 0.3 mM, the EOF was totally reversed, and a stable migration time was observed. But when the concentration of CTAC was increased to 0.5 mM, the peak height of $[Cu–EDTA]^{2-}$ went low, and the peak tailed noticeably. Considering the critical micelle concentration of CTAC [27,28], the concentration of CTAC in the running buffer was finally chosen as 0.3 mM.

3.4. Optimization of FESI

In order to lower the detection limit of $[Cu–EDTA]^{2-}$ with UV detector, the FESI technique was used to make $[Cu–EDTA]^{2-}$ concentrated in the injection step. The injection time and injection voltage were important in FESI. A longer injection time or a higher injection voltage could make a larger injection amount of $[Cu–EDTA]^{2-}$, which caused a higher sensitivity for the detection. But when the injection went overloaded, the peak would broaden. Four FESI times, 4, 5, 6 and 9 min, were chosen to observe their effect, while the injection voltage was fixed at +2 kV. Their electropherograms are shown in Fig. 4. Taking the sensitivity, peak shape and detection efficiency into account, a +2 kV for 5 min was chosen as the optimal injection parameter. During a FESI step, a water plug added was proven to be helpful to improve the focusing effect. Moreover, the water plug was also a big help to improving the reproducibility. When the water plug was set as 0.5 psi for 10 s, a single symmetric peak with little tailing was obtained, and the peak area would not increase when the water plug got longer. The concentration of EDTA in Cu–EDTA solution was another important factor which would affect the FESI efficiency. A low EDTA concentration would cause an incomplete Cu–EDTA complexation. But excessive concentration of EDTA would lead to a too high ionic strength of Cu–EDTA solution, which could in turn affect the injection efficiency of $[Cu–EDTA]^{2-}$. In order to get a good stacking efficiency and hence a low LOD, the concentration of EDTA was set as 1.0 μM to make the ionic strength of Cu–EDTA solution low enough.

3.5. Calibration curve, linear range, detection limit and reproducibility

The calibration curve of $[Cu–EDTA]^{2-}$ was obtained under the optimum conditions. The curve was $y=-17.0+35.1x$, and the

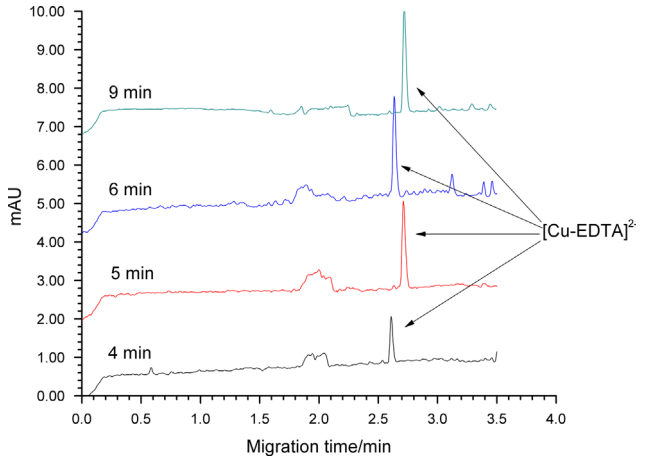

Fig. 4. Effect of the FESI time. Running buffer: 50 mM ABS (pH=5.5) mixing with 0.3 mM CTAC. Sample solution and other experimental conditions the same as in Fig. 2.

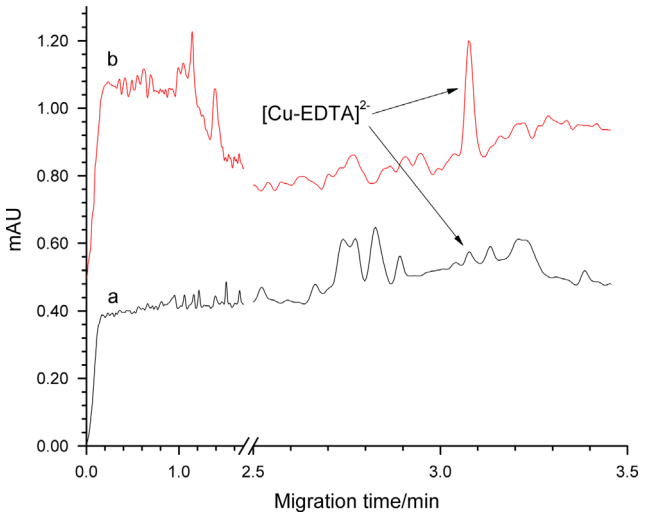

Fig. 5. Electropherograms of HeLa cells extract. (a) Blank HeLa cells extract and (b) 20 μM Cu-LL3-31 treated HeLa cells extract. Other experimental conditions the same as in Fig. 2.

correlation coefficient of the curve was 0.9987. In this curve, y represented the peak area of $[Cu–EDTA]^{2-}$ (μAU s), and x as the concentration of Cu^{2+} (nM). The linear range was from 10 nM to 500 nM, with the LOD ($S/N=3$) of 2.7 nM. The relative standard deviations ($n=5$) of the migration time and the peak area were 0.55% and 7.2% at 10 nM Cu^{2+}, respectively.

3.6. Application to HeLa cells

Two kinds of cell sample solutions extracted by two methods including acid-hydrolysis and freeze–thaw, were used to assess the accuracy of the proposed CE^2–FESI method. The samples were filtered through a 0.45-μm polytetrafluoroethylene membrane filter before CE_1 running.

Before the solution extracted by acid-hydrolysis was injected into the capillary, it was 1:1 diluted with 2 μM EDTA to keep the concentration of EDTA the same as that used in the calibration curve. But a very poor electropherogram was obtained for the CE^2–FESI detection. A giant and broad peak appeared, and it was supposed to fully cover the peak of $[Cu–EDTA]^{2-}$, which made $[Cu–EDTA]^{2-}$ hardly be detected. The giant peak was proven as the peak of residual H_2O_2 in the extract experiment. Evidently, the H_2O_2 used to dissolve the sulfides could not be fully eliminated by heating at 90 °C for 30 min. With the help of EOF, the H_2O_2 in sample could be injected into the capillary even though it hardly ionized in the solution. Adding some catalysts such as Fe^{3+} could eliminate the residual H_2O_2 more completely, but it might further increase the ionic strength of sample solution, leading to a worse stacking efficiency. Taking into consideration, such acid-hydrolysis method was abandoned.

Since FESI has a limitation for the ionic strength, an almost purely physical extraction method was developed with only EDTA used, i.e. the freeze–thaw method (Fig. 1). Before the sample solution extracted by this method was injected into the capillary, the solution was diluted 100-fold by pure water to adjust the concentration of EDTA to 1 μM. Both blank cells and Cu-LL3-31 treated cells were used for the determination. As shown in Fig. 5a, the $[Cu–EDTA]^{2-}$ peak is so small that it seems a baseline fluctuation. The original Cu amount in blank cells was calculated as 3.1 nM based on calibration curve, which has approached the LOD of the proposed method and is not in good accordance with ICP-AES result (7.9 nM). Additionally, for Cu-LL3-31 treated cells, the amount of Cu were 14 nM, showing the interaction between cell and Cu-containing drug. The difference of detection results between the proposed CE^2–FESI and ICP-AES may be owing to the following reasons: (1) the detection value of 3.1 nM has been out of linear range (10–500 nM) and in a way the "real" value might be between 2.7 nM and 10 nM; (2) the sampling of cells has uncertainty, and it is hard to quantitate biologically, unlike with respect to pure chemistry; (3) the coordination equilibrium among EDTA, copper and proteins cannot make the copper be fully extracted out of cell just by EDTA; and (4) other metals in cell could also form chelates with EDTA, which further decreased the extraction efficiency of copper. Consequently, the proposed CE^2–FESI still needs further investigation.

4. Conclusions

This study established a fast, simple and highly sensitive CE_1 method to detect copper with EDTA as a complexing agent. Using FESI as the online-concentration method, an LOD of 2.7 nM was obtained. Two different methods, acid-hydrolysis and freeze–thaw, were used to extract copper out of cell. But only the extract using freeze–thaw could be applied in the determination. The application of such a CE^2–FESI method proved its promising value in cell analysis.

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